

# Mechanical force-induced DNA damage during AFM single-molecule manipulation

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**Abstract** Many environmental factors can cause DNA damage, such as radiation, heat, oxygen free radical, etc., which can induce mutation during DNA replication. Meanwhile, DNA molecules are subjected to various mechanical forces in numerous biological processes. However, it is unknown whether the mechanical force would induce DNA damage and introduce mutation during DNA replication. With the combination of single-molecule manipulation based on atomic force microscopy (AFM), single molecular polymerase chain reaction (SM-PCR) and Sanger's sequencing, we investigated the effect of mechanical force on DNA. The results show that mechanical force can cause DNA damage and induce DNA mutation during amplification.

**Key words** Manipulation, Atomic force microscope, Polymerase chain reaction (PCR), DNA damage and mutation

## 1 Introduction

Mutations are a series of small changes in a genomic sequence. DNA mutation is not only the driving force of biological evolution but also the theoretical basis of current breeding techniques. One important source of mutation is the intrinsic inaccuracy of polymerase in the replication process. The other is physical or chemical damage to the genetic materials<sup>[1]</sup> that originates endogenously<sup>[2]</sup> and exogenously<sup>[3]</sup>, including radiation, heat, oxygen free radical, etc., which induce mutation during subsequent replication<sup>[1]</sup>. Recent progresses in molecular biology and mutagenesis techniques have provided possibility to induce the change of genetic materials<sup>[4]</sup> and detect the genetic changes qualitatively<sup>[5,6]</sup>. For example, radiation breeding utilizes the introduction of genetic material changes to generate mutants with desirable traits<sup>[7]</sup>. Detection of genetic changes can be applied for cancer diagnostics<sup>[8]</sup> and supervising the radiology dose.

Most of genetic changes are fatal for cells and

lead to the failure of research of mutation based on cell culture, which hinder the detection of genetic changes. To get all the mutations statistically, it is necessary to conduct the experiment *in vitro* with single molecule, both in replication and mutation detection. Therefore, the analysis of DNA mutation based on a single DNA molecule is of critical importance for the fundamental genetic research and industrial biotechnology development, such as radiation breeding and radiology dose supervising.

Manipulation of individual atoms and molecules is emerging as an important frontier in physical research<sup>[9,10]</sup>. With the development of new experimental tools, e.g. optical tweezers and scanning probe microscope, manipulation of single biomolecules such as DNA or proteins has become possible<sup>[11]</sup>. Plenty of researches on single DNA molecule focus on its mechanical properties<sup>[12]</sup>. The atomic force microscope (AFM) can image and manipulate native biological samples on surfaces at the single-molecule level<sup>[13,14]</sup>, and the applied mechanical force by AFM to the biological samples

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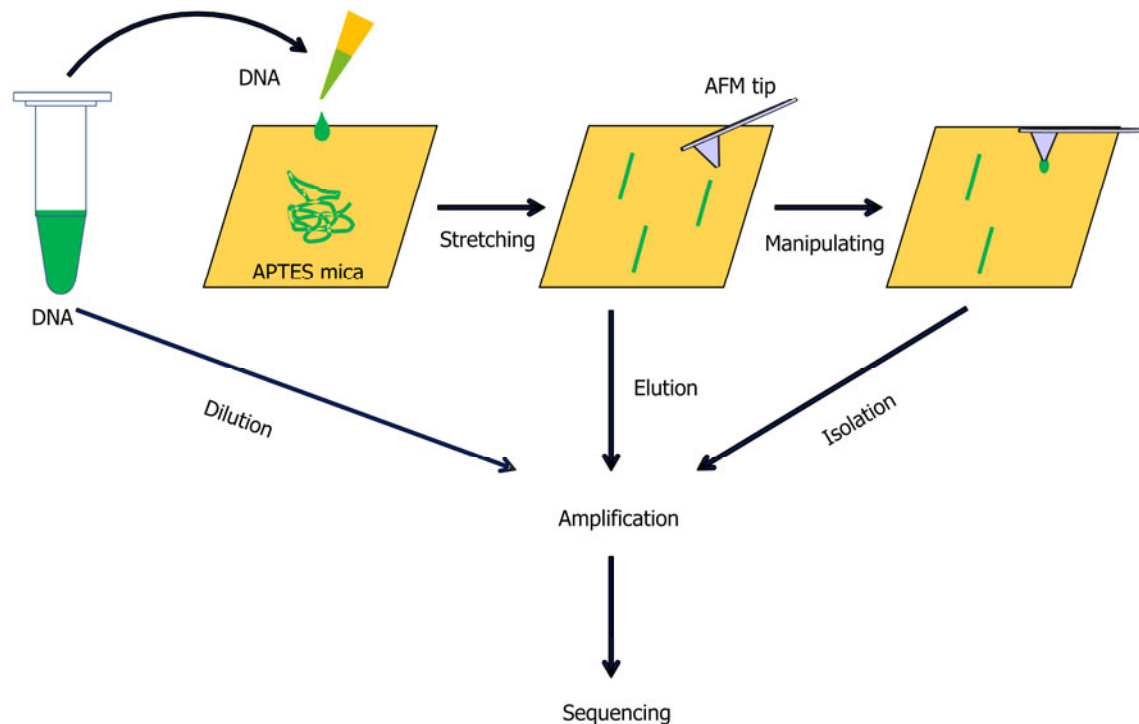
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can be controlled in a precise manner<sup>[15,16]</sup>. Recently, we have developed a novel strategy for ordered single-molecule sequencing, which combines AFM-based manipulation technique, subsequent single-molecule polymerase chain reaction (PCR) and Sanger's method<sup>[17,18]</sup>.

However, it is still unknown whether or how the DNA molecules would be damaged by such mechanical force during manipulation. In fact, *in vivo*, DNA molecules are subject to mechanical force in numerous biological processes<sup>[12]</sup>, such as DNA transcription, gene expression and DNA replication. For example, DNA helicases must generate force to

unzip the parental DNA strands during replication. In these processes, whether the mechanical force will lead to DNA damage and mutation is still unclear. Thus, it is important to set up a technique that can detect the DNA damage and mutation under a certain force.

In this paper, we establish a method and investigate the DNA mutations induced by mechanical force by using combined techniques, including single-molecule manipulation based on atomic force microscopy (AFM), single molecular polymerase chain reaction (SM-PCR) and Sanger's sequencing.



**Fig.1** Schematic representation of amplification and sequencing of DNA with different treatments, including diluting, stretching and manipulating, respectively. DNA molecules used in this experiment were 569 bp in length.

## 2 Materials and methods

### 2.1 Materials and instruments

The template pBR322 plasmid DNA and *ExTaq* Hotstart DNA polymerase were obtained from TaKaRa Bio. Inc. (Dalian, China). Two sets of primers used for amplification were synthesized by Sangon Company (Shanghai, China). PCR procedure was carried out using Eppendorf Mastercycler Gradient (Eppendorf Inc., Germany). Imaging and manipulation of DNA were performed with a multimode AFM (Nanoscope IIIa, Veeco/Digital Instruments, Santa Barbara, CA)

using the tapping mode and lift mode in air. Silicon cantilevers (NSC11, NSC35-Pt/Ti) were employed. APTES (3-aminopropyl triethoxysilane) used for mica modification was purchased from Sigma-Aldrich.

### 2.2 Sample preparation, AFM imaging and manipulation

The DNA molecules used for manipulation, 569 bp (base pairs) in length, were amplified by PCR system, with the upstream primer (P1: 5'-GTCGTTTGGTATG GCTTCA-3') and downstream primer (P2: 5'-GAC AATAACCCTGATAAATGCT-3'). The product

(pBR322 original sites from 3632<sup>th</sup> to 4200<sup>th</sup>) was purified with the TaKaRa DNA fragment purification kit. After diluting to appropriate concentration with TE buffer, the DNA fragments were deposited and stretched by a modified “molecular combing” technique<sup>[19,20]</sup> onto a pretreated APTES-mica substrate<sup>[21]</sup>, as shown in Fig.1.

Imaging and manipulating operation of DNA were performed with a multimode AFM and silicon cantilevers in air condition. In manipulating operation, the AFM tip was lowered down on to the surface and scanned across the target DNA molecule. Then, the target DNA molecule on mica was picked up (isolated) by AFM tip, as shown in Fig.1.

### 2.3 PCR amplification of DNA samples

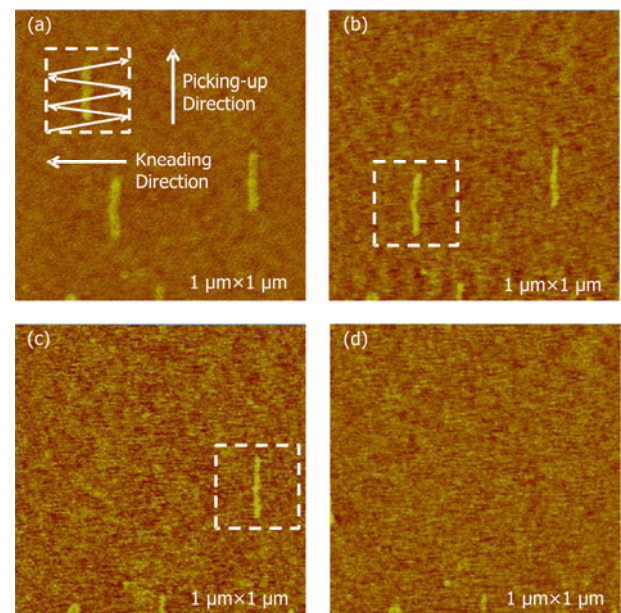
Single or several isolated DNA fragments 569 bp with the AFM tip were transferred into a sterilized PCR tube and amplified by single-molecule PCR as shown in Fig.1. Meanwhile, in order to confirm no contamination from AFM tips, an unused tip was used as the PCR negative control. The 569 bp DNA was used as templates for positive control, while Milli-Q water as another negative control.

As control experiments, diluted DNA solution was used as PCR templates. In addition, DNA molecules were first stretched on APTES-mica<sup>[20,21]</sup> and then eluted with Milli-Q water. The eluted DNA was diluted and also used as PCR templates (Fig.1).

### 2.4 Analysis of PCR products and DNA sequencing

PCR were carried out with the upstream primer (AP1: 5'-CTCTTACTGTCATGCCATCCG-3') and downstream primer (AP2: 5'-CCGTGTCGCCCTTAT TCC-3'). This set of primers bind to the template just inside the first set of primers (P1 & P2), resulting in a product of 338 bp in length. The PCR procedure was as follows: 5 min at 95°C for pre-denaturation, followed by 40 cycles of amplification: 30 s at 95°C, 30 s at 63°C, and 30 s at 72°C. After a final elongation step of 72°C for 5 min, PCR tubes were maintained at 4°C. The products were analyzed by agarose electrophoresis and stained by ethidium bromide. The positive products were sequenced with the PCR primers (AP1 & AP2) using an ABI 3700 automated

DNA sequencer. The sequencing results were analyzed by Bioedit Software.



**Fig.2** Imaging and manipulating of DNA molecules on mica substrate, (a) AFM image of DNA molecules, the DNA molecules aligned on substrates along a chosen direction; the white frame: during single-molecule manipulation, the AFM tip was lowered down onto the mica surface and scanned across the target DNA, the DNA molecule was kneaded from one of its end to the other. (b) AFM imaging was performed after manipulation operation. The target DNA molecule disappeared from the mica surface, indicating it was picked up successfully by the AFM tip. (c) and (d) Subsequently, other DNA molecules were picked up by the AFM tip successively with the same procedures.

## 3 Results and discussion

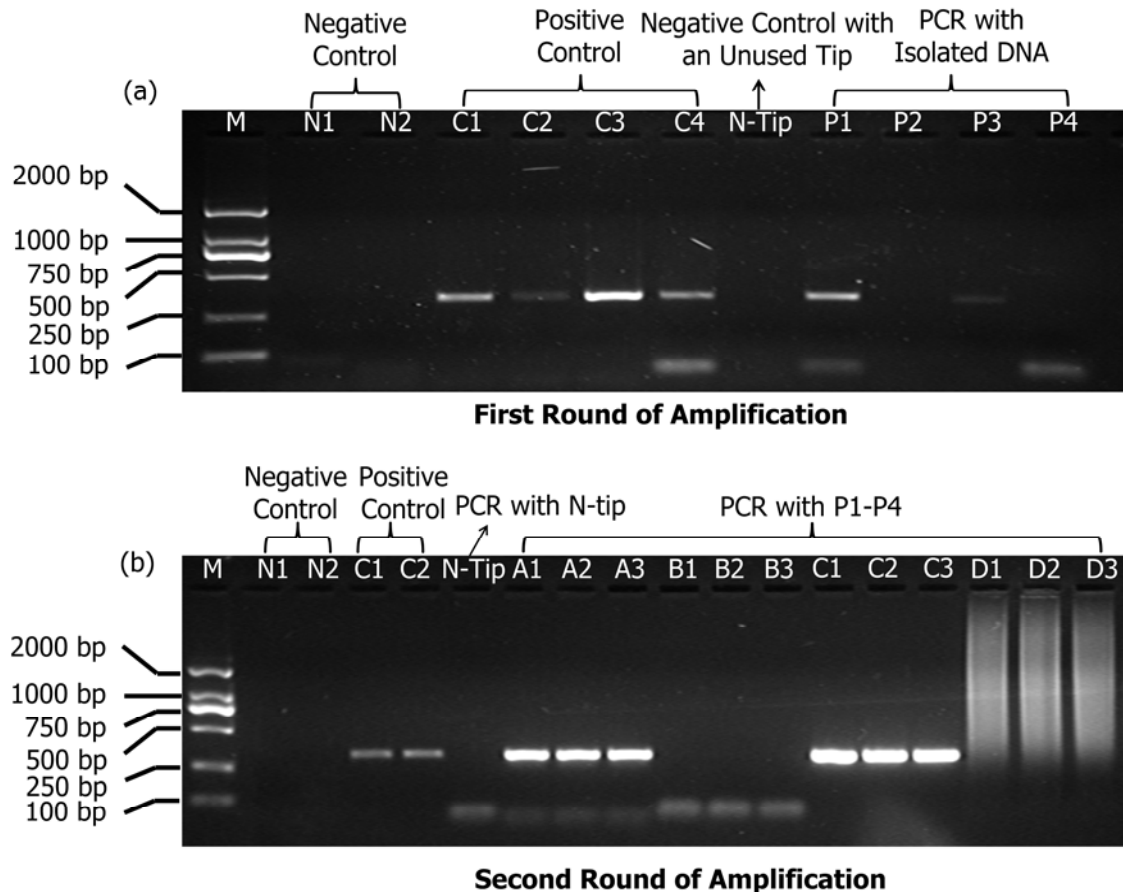
### 3.1 Imaging and manipulation of DNA molecules on mica substrate

Prior to manipulation, AFM imaging in tapping mode was usually taken in a large scan size. As shown in Fig.2a, DNA molecules were aligned on APTES mica substrate along a chosen direction by using the modified “molecular combing” technique. The orientation of the aligned DNA molecules depends on the direction of the fluid flow during the process. After a desired area was chosen, the scan size was zoomed to 300 nm×300 nm (white frame in Fig.2a) to precisely localize the picking-up site on the target DNA molecule. The following picking-up process is carried out in the manipulation mode. As shown in white frame of Fig.2a, when the AFM tip scanned across the target DNA molecule, the DNA molecule was kneaded

from one of its end to the other by the AFM tip.

After picking-up process, the imaging mode was switched back to a large scan size for verifying the result of manipulation. As shown in Fig.2b, the target DNA molecule was disappeared from the

surface, indicating it was picked up by AFM tip. Subsequently, other DNA molecules were picked up by AFM tip successively with the same procedure, as shown in Figs.2c and 2d.



**Fig.3** Electrophoresis analysis of single-molecule PCR. M denotes DL2000 DNA marker. The target band is 338 bp in length. (a) Products of first round of amplification. Both of the negative controls with the absence of template DNA (N1 and N2) display target band, indicating no contamination in the PCR system. All of the 4 positive controls (C1–C4) show successful amplification of the template DNA. The result of negative control with an unused tip indicates there is no contamination on AFM tip. P1–P4 indicate amplification with isolated DNA. Positive results with target bands of P1 and P3 indicate that the isolated DNA could be successfully amplified. (b) Products of second round of amplification. A–D groups show the products of the second round PCR with isolated DNA by a tip. Every group contains 3 parallel samples. A and C groups show sharp and bright target bands.

### 3.2 Gel electrophoresis analysis of PCR products.

After single molecule PCR, the products of amplified DNA were analyzed by 2% agarose electrophoresis (Figs.3a and 3b) and stained by ethidium bromide. In this experiment, 2 of blank controls with Milli-Q water (N1 and N2 in Fig.3a) and 1 negative control with an unused AFM tip (N-tip in Fig.3a) yielded negative results, suggesting that contamination was controlled effectively. All of 4 positive controls showed successful amplification of the template DNA (C1–C4

in Fig.3a). As a typical example, 2 of 4 isolated DNA with AFM tip were detected positive after amplification (P1 and P3 in Fig.3a), indicating the isolated DNA could be successfully amplified.

In this study, we found that some of products from the first round of PCR were not sufficient for sequencing, as the weak band of P3 shown in Fig.3a. So second-round amplification was carried out. During the second round of PCR, products of the first round (P1–P4 in Fig.3a) were employed as the template in 3 parallel amplifications. After the second round, 2 groups of the PCR products showed positive results

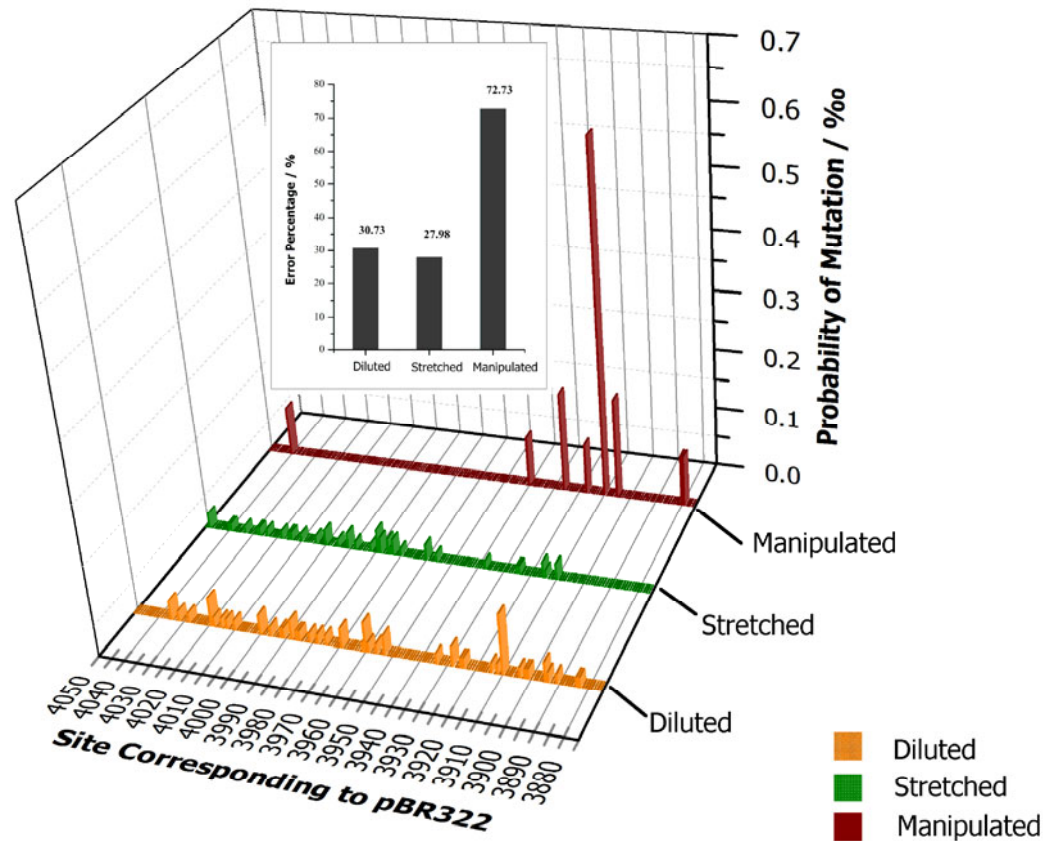


with sharp and bright target bands (A and C groups in Fig.3b), which were subsequently sequenced with the PCR primer (AP1 & AP2) using an ABI 3700 automated DNA sequencer.

### 3.3 Analysis of sequencing results

In total we collected 187 AFM tips containing the picked-up DNA fragments for subsequent PCR. However, due to the low efficiency of single-molecule

PCR, we obtained only 22 successfully amplified and sequenced results. Meanwhile 577 samples (281 for diluted, while 296 for stretched and eluted) without single-molecule manipulation were applied as the controls, which resulted in 403 PCR and sequencing results (180 for diluted and 223 for stretched and eluted).



**Fig.4** Statistical analysis of mutations occurring at every sites of nucleotide. The yellow, green and red columns stand for the case of diluted DNA, stretched DNA and manipulated DNA, respectively. Inset: Percentage of mutations in PCR products with treated DNA as template. The mutation percentage of diluted DNA and stretched DNA were similar, with 30.73% and 27.98%, respectively. In the case of manipulated DNA, the percentage was much higher, with 72.73%, indicating the increase of mutation probability occurring in the sequence after AFM manipulation.

By analyzing the sequencing result we found that single-molecule manipulation could lead an increase of rate of mutation up to ~70%, as shown in inset of Fig.4. In the case of control experiments with diluted and stretched DNA, the rates of mutation were approximate, with about 30%, respectively. No significant differences were found between DNA samples treated by diluting and stretching. The increase in mutation rate indicates that, during manipulating, mechanical force could induce some

kind of damages to DNA molecules and lead to the mutation after PCR amplification.

It was found that, in the case of mechanical-force induced mutations, sixteen single-nucleotide mutations occurred (the red column in Fig.4), including seven mutations at the 3 910<sup>th</sup> site with C>T, two at 3 905<sup>th</sup> site with G>C, while one at the 3 917<sup>th</sup> site (G>A), etc. By contrast, mutations of samples with diluting and stretching occurred randomly (the yellow and green columns in Fig.4).

To describe the mutations in the sequences of DNA samples with different treatments, we calculated the probability of mutations for each site of nucleotide *via* the following formula,

$$P_i = \frac{n_i}{N \times M}$$

where  $i$  is the site index,  $n_i$  is the number of mutations occurring at the  $i^{\text{th}}$  site.  $N=338$  is the number of base pairs in the sequence, and  $M=22$ , for example, is the number of successful experiment with manipulation. Remarkably, we observed an unexpected result in the statistics analysis. In the cases of diluted and stretched samples, the probabilities of mutation at 3 910<sup>th</sup> site were 0.0098% and 0.0026%, respectively (the yellow and green columns in Fig.4). By contrast, the mutation probability at 3 910<sup>th</sup> site of the DNA molecules treated with mechanical force was much higher, with a value of 0.0592% (the red column in Fig.4). These data indicated that DNA samples after our single-molecule manipulation process exhibited a non-random characteristic at 3 910<sup>th</sup> site in sequence of pBR322. Though the sample number for statistics was limited (11.8% or 22/187 for manipulated samples) due to the low success rate in single-molecule PCR, and it is hard to distinguish in our current experiments in which procedure the mutation occurred. Our findings hinted that hot spots might exist in mechanical force induced DNA mutation.

#### 4 Conclusion

In this study, by the combination of AFM single-molecule manipulation, single-molecule PCR and Sanger's sequencing, we successfully established a method to investigate the effect of mechanical force on DNA damage and mutation. The initial experimental results showed that DNA mutation rate could be increased after mechanical manipulation. Future work should be focused on the improvement of the experimental efficiency to offer sufficient data in order to give a conclusive result. By analyzing the mutation results in each procedure, it will be very interesting to explore the possibility of the nonrandom mutation induced by mechanical force.

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